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Review

Supercritical fluid extraction and chromatography for fat-soluble vitamin analysis

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Abstract

Extraction and chromatographic separation of fat-soluble vitamins is a challenging task, due to the sensitivity of these compounds towards light, oxygen, heat and pH. In light of this, supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC) are attractive techniques as they function at considerably milder conditions than conventional solvent-based analytical techniques. Moreover, supercritical techniques consume much less amounts of organic solvents than conventional ones. This review gives a brief description of suitable supercritical media as well as basic theory on SFE and SFC processes. Furthermore, guidelines are provided for optimizing the important extraction and separation parameters to facilitate a successful method development. Finally, applications employing SFE and/or SFC for fat-soluble vitamin enrichment and final determination are reviewed.

Keywords: Reviews; Supercritical fluid chromatography; Supercritical fluid extraction; Extraction methods; Vitamins; Tocopherols; Carotenoids

Contents

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1. Introduction

Fat-soluble vitamins are organic molecules that are nutritionally essential to the human body [1]. Lack of vitamins can lead to serious diseases such as night blindness (vitamin A), rickets and weakening of bones (vitamin D), rupturing of blood cells and cancer (vitamin E) and blood coagulation diseases (vitamin K). However, over consumption of vitamins can also be dangerous [1–4]. These factors have resulted in strict regulations for vitamin use in food nutrition, requiring the need for continuous analysis of food products and pharmaceutical preparations. New methods are constantly being developed regarding the extraction and enrichment of fat-soluble vitamins from natural sources such as plants, oilseeds and vegetables. For example, carotenoids (vitamin A) are used as antioxidants and natural pigments, to enhance the value of the food products and to provide color ranging from yellow and orange to red. Tocopherols (vitamin E) are also commonly isolated from plants and oilseeds, and often added as natural antioxidants to foods and dietetic products. Moreover, vitamins A and E are thought to prevent skin damage, and are commonly found as additives in cosmetic creams and ointments.

The conventional method for the isolation of fat-soluble vitamins from a sample matrix includes solvent extraction, using solvents such as hexane, ethanol, acetone, methanol, tetrahydrofuran and petroleum ether [5,6]. Complex sample matrices, such as food products and animal feeds, are usually saponified prior to extraction in order to disrupt the matrix and degrade triacylglycerols to glycerol and produce soaps of the free fatty acids [7–9]. Solid phase extraction (SPE) has been employed in some applications [10,11], which also is a technique requiring relatively large amounts of organic solvents. Analytical determination of the vitamins is commonly achieved by normal-phase or reversed-phase liquid chromatography (LC) using UV–Vis or fluorescence detection [7,9,12]. Gas chromatography (GC) with flame ionization (FID) or mass spectrometry (MS) detection has also been employed, even though the thermolabile vitamins generally require derivatization prior to GC [13].

Fat-soluble vitamins are sensitive towards exposure to extreme pH, oxygen, light and heat, which complicates both sample preparation and subsequent separation [2]. Thus, conventional sample preparation techniques are not always ideal for fat-soluble vitamin analysis. Saponification operates at extremely high pH, causing partial isomerization of vitamin D and total degradation of vitamin K. Conventional sample preparation techniques usually include heating of the sample/solvent system, in order to shorten

the extraction time. Overall, the use of organic solvents in fat-soluble vitamin applications requires extensive degassing and the use of high purity of the solvents, protection from light and excessive heat, and inclusion of protective antioxidants. In addition, conventional solvent-based extraction techniques are time-consuming and labor intensive. LC consumes large volumes of organic solvents, which are occasionally chlorinated solvents. Utilization of GC creates a high risk of thermal degradation of the vitamins, even when derivatization is done prior to GC separation.

The use of supercritical fluids (SCFs) in fat-soluble vitamin analysis provides an interesting alternative to the use of organic solvents. The main advantages of using supercritical fluids instead of conventional organic solvents are the minimal consumption of organic solvents, the exclusion of oxygen, and the reduction of heat. Modern supercritical fluid extraction (SFE) offers shorter extraction times, potentially higher selectivity and increased sample throughput (due to available automated instruments) compared to conventional solvent extraction techniques. The chromatography analogue supercritical fluid chromatography (SFC) permits the separation of compounds of widely different polarities and molecular masses, and eliminates need for derivatization of the fat-soluble vitamins. In theory and most often also in practice it is faster than LC, due to higher mass transfer rate in the chromatographic process.

This review reports on the analysis of fat-soluble vitamins employing supercritical techniques (SFE and SFC), including both preparative and analytical applications. There are several excellent review articles on the development of SFE methodologies and application within different areas of analytical chemistry [14–17], including the analysis of food [18] and plant products [19], however, coverage regarding fat-soluble vitamin analysis has been limited.

1.1. What is a supercritical fluid?

The critical point of a fluid (C_p) is defined by its critical pressure (P_c) and temperature (T_c). At temperatures above the critical point, only one state exists, the supercritical fluid state. Here, the distinction between the gas and the liquid phases has disappeared, and the resulting supercritical fluid has one uniform density [20]. With increasing pressure, the density of a supercritical fluid approaches that of a liquid solvent, giving it solvent properties equivalent to those exhibited by liquid media. In addition, supercritical fluids possess gas-like viscosities, which accelerates analyte mass transfer. Hence, supercritical fluids can easily penetrate different types of sample matrices in most SFE applications, and can provide higher separation efficiency in SFC applications relative to LC. Furthermore, the density of supercritical fluids and hence the solvent strength is easily changed by varying pressure and/or temperature. This enables the use of one fluid for various applications, without using multiple liquid solvents.

Compounds, which have been used in their supercritical state, consist mainly of carbon dioxide, nitrous oxide, ethane, propane, *n*-pentane, ammonia, fluoroform, sulphur hexafluoride and water. These are listed in Table 1 along with their critical parameters.

Supercritical carbon dioxide (SC-CO_2) is the most frequently used supercritical fluid in fat-soluble vitamin applications as well as in other applications, due to its (i) low T_c and P_c, (ii) chemically inertness, (iii) low cost, and (iv) low toxicity and non-flammability. The major disadvantage in employing SC-CO_2 is its nonpolar character, which sometimes necessitates the addition of smaller amounts of a polar organic modifier, such as methanol. There are only a few reported applications on the use of other fluids than SC-CO_2, such as the use of subcritical propane for extraction of tocopherols and carot-

Table 1
Properties of some supercritical fluids at the C_p [22]

supercritical fluid	T_c (°C)	P_c (bar)	Critical density (g/ml)
CO_2	31.0	72	0.47
N_2O	36.5	70.6	0.45
Ethane	32.3	47.6	0.2
Propane	96.7	42.4	0.22
n-Pentane	196.6	32.9	0.23
Ammonia	132.5	109.8	0.23
Fluoroform	26	46.9	0.52
SF_6	45.5	38.0	–
Water	374.2	214.8	0.32

enoids. These applications will be discussed below (see Section 2.3).

2. Supercritical fluid extraction

A flow schematic of the basic SFE equipment is shown in Fig. 1.

The principle of SFE is quite simple. Samples are weighed into extraction cells, the extraction chamber is heated and pressurized to a set value, and the supercritical fluid is then pumped through the extraction cell facilitating extraction of the target analytes from the sample matrix. The extract flows through a restriction device, where the pressure is reduced to ambient. The supercritical fluid expands rapidly to the gaseous state (if the supercritical fluid is a gas at ambient conditions), and the analytes are trapped in the collection device. Collection can be performed on a solid-phase trap, in an empty vessel, in a vessel tube containing a solvent, or may even be cryogenically focused in a capillary for on-line coupling with a chromatograph. When a solid-phase trap is utilized, it is frequently rinsed with a small quantity suitable solvent into a collection vial.

There are several excellent books [20–24] and articles [17,25,26] describing SFE theory and instrumentation, which the reader can consult.

2.1. Important parameters affecting solubility in supercritical fluids

2.1.1. Pressure

Increasing pressure of the supercritical fluid at constant temperature results in increasing density, and thereby higher solvent strength for the supercritical fluid. One way of describing this relationship is to use a modified version of the Hildebrand equation (Eq. (1)), which gives the solvent strength (Hildebrand parameter, δ) as a function of the reduced density of the supercritical fluid (ρ_{sf}) with respect to the reduced density of a typical fluid in liquid state (ρ_l), and the critical pressure of the fluid (P_c) [27]:

$$\delta = 1.25 P_c^{1/2} (\rho_{sf}/\rho_l) \quad (1)$$

Theoretically, when the density of the supercritical fluid is equivalent to the density of the target analyte, the maximum solubility of the analyte is achieved in the supercritical fluid.

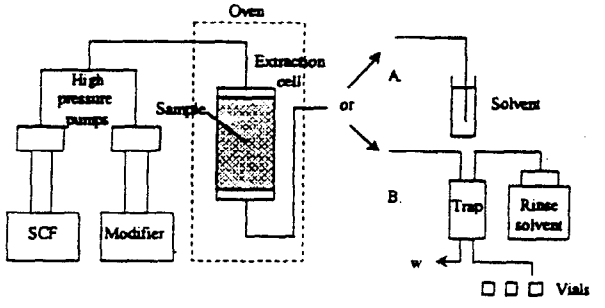


Fig. 1. A simple flow schematic of a supercritical extractor, showing two types of collection modes: (A) solvent collection and (B) solid-phase trapping.

2.1.2. Temperature

When pressure of a supercritical fluid is constant and temperature is increased, the effect on the solvent strength depends on the pressure [28]. If this pressure is below the "crossover point", increasing temperature leads to lower solvent strength of the fluid due to the decrease in fluid density. Above the "crossover point", an increase in temperature can improve the extraction efficiency despite the decrease in fluid density, since the vapor pressure of the analyte is increased. The "crossover point" depends on analyte–supercritical fluid interactions, and has been reported for several solutes in SC-CO_2, including triacylglycerols [29], plant-derived oil [30] and carotenoids extracted from sweet potatoes [31].

2.1.3. Modifiers

A modifier may be added to SC-CO_2 in order to improve the solubility of the analytes. Normally, the modifier should be a good solvent for the analytes, and ethanol [32–34], propane [30], hexane [34–36], dichloromethane [34] and chloroform [36] have all been used for the extraction of carotenoids and tocopherols from plants and vegetable matrices, and methanol has been used for the extraction of vitamins A and E from dairy and meat products [37,38].

2.1.4. Analyte solubility data

Solubility data for a range of different lipophilic compounds have been determined. Such data exist for sterols [28], retinyl palmitate [39], α-tocopherol

[40,41], vitamins A, D, E and K [42] and β-carotene [43] in SC-CO_2 at different temperatures and pressures. Catchpole et al. [39] showed that the solubility behavior of retinyl palmitate and squalene in SC-CO_2 was typical to that exhibited by other lipids, and noted that a linear relationship exists between the solute solubility expressed on a logarithmic scale versus the density of SC-CO_2. It has also been demonstrated that the solubility of squalene increases linearly with the increasing ethanol concentration of the supercritical fluid.

Johanssen and Brunner [42] measured the solubilities of vitamins A, D, E and K in SC-CO_2 at temperatures between 40 and 80°C and at pressures of 20–35 MPa. Their studies demonstrated that the solubility of all components increased with increasing density at constant temperature. Furthermore, at constant pressure, the solubility increased with increasing temperature for vitamins A, D and E. Vitamin K_1, however, showed a decrease in solubility with increasing temperature, presumably due to the much lower vapor pressure of vitamin K. For vitamin D_2 the crossover pressure was recognized to lie between 21 and 22 MPa. Overall, the solubility for vitamins A, D, E and K was approximately 10 g/kg, except for β-carotene, which was about 3 orders of magnitude less soluble than those vitamins.

However, solubility alone is not sufficient when developing an extraction method on real samples. For example, in many food products, fat-soluble vitamins are found along with triacylglycerols and sterols, which then also might need to be solvated in the supercritical fluid in order to obtain a quantitative extraction of the fat-soluble vitamins. In a study by Stoldt and Brunner [44], solubility data of individual lipid compounds were determined from complex mixtures containing triacylglycerols, diacylglycerols, free fatty acids, sterols, tocopherols and carotenoids employing SC-CO_2. It was shown that the solubility of a component in SC-CO_2 is significantly dependent on the composition of the lipid sample, thus the coextractives can be thought of acting like "solubility-enhancing" modifiers.

2.2. Method development

The dynamic extraction process of an analyte from a sample matrix can be divided into four steps, i.e., (i) supercritical fluid penetration into the sample matrix; (ii) reversible release of the analyte from the matrix; (iii) diffusion of the dissolved analyte to the edge of the matrix particle; and (iv) final removal of the analyte by solvation into the bulk supercritical fluid [45]. Therefore, in order to obtain highest possible extraction efficiency, it is not only the solubility of the solute in the supercritical fluid which needs to be considered, but also the type of sample matrix being extracted and the magnitude of the analyte–matrix interactions. Different sample types will also require different kinds of sample pre-treatments, as well as different extraction conditions and an initial static versus dynamic extraction mode, as well as variability in total extraction time.

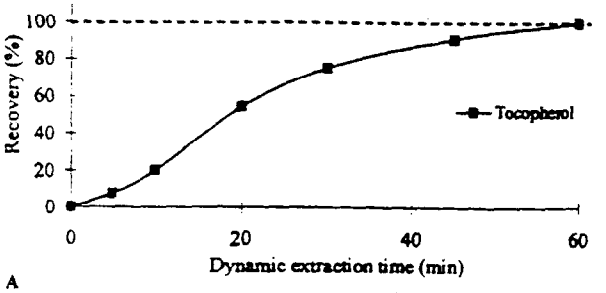
2.2.1. Classification of the sample

In order to facilitate the optimization of extraction parameters for fat-soluble vitamins, the sample should be classified regarding: (i) the chemical and morphological nature of sample, e.g., fibrous, starchy etc; (ii) the forms in which the vitamins is present, e.g., free, esterfied or complexed to the matrix components; (iii) concentration of the vitamin; (iv) the water content of the sample; and (v) the fat content of the sample. The method development can be simplified by dividing the extraction process into two steps: (i) removal of the analytes from the matrix to the surface of the matrix particle, and (ii) solvation of the analytes in the supercritical fluid and transport to the collection device [24]. The first step can be viewed as an irreversible desorption/diffusion process and the second step can be viewed as a chromatographic (reversible) elution process and is mainly governed by the solubility of the analytes in the supercritical fluid. Both these steps govern the rate of the extraction, but the former step is usually the rate limiting one. Hence, in order to optimize the analytical procedure, the effect of SFE flow-rate on the extraction rate should be tested on a particular sample matrix, to determine whether the extraction is desorption- or solubility-controlled [46].

Desorption-controlled matrices usually occur where strong interactions between matrix and analytes exist. Moreover, the analytes could be enclosed by a hard structure, such as fat globules in milk, or lactose shells in milk powder, which will make the analytes relatively inaccessible. Therefore, the ex-

traction rates of analytes from such matrices are desorption-controlled, and not affected much by fluid flow-rate. Grinding of the sample, increasing the extraction temperature and adding a modifier are all suitable means for facilitating desorption of analytes from the sample matrix [24,46]. In these cases a relatively long static extraction step is often included to decrease the consumption of extraction fluid and improve the extraction efficiency. For example, in Fig. 2a the extraction of vitamin E-type components from infant formula demonstrates a typical example of an analyte desorption-controlled extraction process. Here, it is obvious that the dynamic extraction process is slow, and the 15-min static followed by a 60-min dynamic extraction aids in obtaining quantitative recovery of vitamin E from infant formula.

For analytes where extraction is solubility-controlled, the analyte–matrix interactions are usually weak. Hence, the extraction rate mainly depends on



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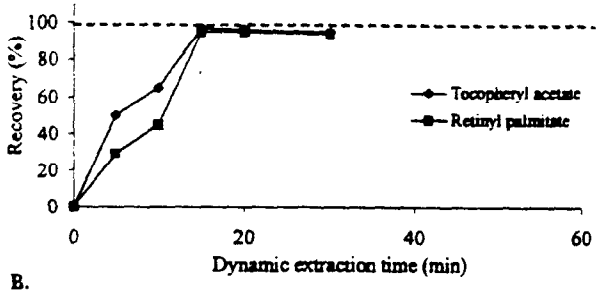

B.

Fig. 2. (A) Extraction of α-tocopherol from infant formula (standard reference material SRM1846 from National Institute of Standards and Technology (NIST). SC-CO_2 containing 5% ethanol was used at 260 bar and 60°C. A 15-min static extraction was followed by a 60-min dynamic extraction at 0.5 ml/min flow-rate. (B) Extraction of α-tocopheryl acetate from a tablet preparation [47]. SC-CO_2 at 250 atm and 40°C using the dynamic extraction mode at a flow-rate of 190–220 ml/min (measured as gaseous CO_2, which corresponds to ca 0.7–1.0 ml/min of SC-CO_2).

the partitioning of the analytes between the matrix and the supercritical fluid, and is therefore increased by using higher flow-rates [46]. Increasing solvent strength (i.e., density) of the supercritical fluid and using smaller sample sizes will aid in completing the extraction [24,46].

Fig. 2b demonstrates a typical example of a solubility-controlled SFE process for the extraction of vitamin esters from powdered tablets [47]. Here, quantitative extraction of tocopheryl acetate and retinyl palmitate was achieved within 15 min using just dynamic extraction, employing neat SC-CO_2 at 40°C and 250 atm (1 atm = 101 325 Pa). This quick SFE is undoubtedly facilitated by the large total surface of the sample in contact with the supercritical fluid, due to the powdered nature of the sample matrix.

2.2.2. Sample pre-treatment

Sample pre-treatment in this work and related publications is defined as any treatment to the sample prior to SFE. For example, all samples should be well homogenized to obtain representative sub-samples. This is particularly important in analytical SFE where sample sizes are frequently small (typically around 0.5–10 g). It is also important to ensure that the particle size is as small as possible. This has been shown to enhance the extraction efficiency by shortening diffusion distances for the analytes [31,34,48,49]. The addition of an antioxidant to the sample and the exclusion of light during the sample pre-treatment procedure are also critical means to minimize vitamin degradation.

Water in the sample can either facilitate the extraction by acting as a modifier or hinder it by blocking the contact with the supercritical fluid [24]. Water can also lead to hydrolysis and degradation of the analytes due to the increasing acidity in the water caused by absorption of CO_2 [50]. In addition, if a solid-phase trap is used, it may be deactivated because of the adsorption or condensation of coextracted water. However, this can be avoided by using relatively high trap temperatures, although there is an inherent risk of thermal degradation to the fat-soluble vitamins. Therefore, samples containing large amounts of water, for example milk, may require freeze-drying before extraction. In general, freeze-dried samples result in higher extraction efficiency

than hydrated samples, as demonstrated for α-tocopherol extraction from palm leaflets [35] and β-carotene extraction from sweet potatoes [31]. However, as noted by Goto et al. [48], it is more important to disrupt the sample matrix into smaller particles when freeze-dried samples are used than when using fresh samples.

Air-drying should be avoided, since it can cause severe oxidation of the vitamins [31,35]. Samples containing smaller amounts of water only require mixing with a drying agent, such as basic alumina, molecular sieves, magnesium sulphate or Hydromatrix. Caution has to be taken to avoid loss of analytes by adsorption on the drying agent [24]. The sorbent Hydromatrix has turned out to be very efficient at adsorbing excess water as it contains hydrophilic groups [24], and has been widely used in analytical SFE [34,37,38,51–54]. In addition, this material increases the contact surface between the sample and the supercritical fluid.

When the sample mixture is loaded into the extraction cell, it should be completely filled, since excess dead-volume may result in an inefficient extraction. If an entrainer is added to the sample before the extraction, it is advantageous to employ a delay time prior to SFE, in order to enhance the desorption effect of the solvent. For example, it was found that when 2 ml of methanol or ethanol was added to 0.5-g milk powder samples, a 3-h delay time instead of only 1 h increased the recoveries of vitamins A and E by almost 40% [53].

Finally it should be noted that the addition of water is required to obtain matrix modification of some samples, such as infant formula [9]. This matrix is difficult to disrupt, and equal amounts of hot (~90°C) water and sample are commonly mixed prior to extraction in order to obtain quantitative recovery of the fat-soluble vitamins [55].

2.2.3. Extraction conditions

Development of SFE methodologies for fat-soluble vitamins requires careful optimization of the extraction temperature. Increasing the temperature may facilitate desorption/diffusion and improve the extraction rate, or alternatively it may worsen the extraction rate due to resulting decrease in solvent strength of the supercritical fluid (if the pressure is below the "crossover point"). An increase in the extraction pressure will on the other hand almost always be beneficial, since it improves the fluid's density i.e., the solvent strength of the extraction fluid. However, the density of the fluid should not be higher than necessary to obtain quantitative recovery of the analytes, since higher fluid densities generally lead to lower selectivity of the analyte during SFE. For example, it has been demonstrated that higher pressure results in poorer selectivity for the extraction of tocopherols and carotenoids from palm oil components, due to increasing solubility of triacylglycerols [56]. Favati et al. [57] have shown that by carefully optimizing the pressure, a high selectivity of carotene extraction from leaf protein concentrates could be obtained with regards to the less soluble lutein. The effect of temperature on selectivity has also been studied by Skerget et al. [58] for the fractionation of aromatic components and carotenoids from paprika. In this study it was found that a lower extraction temperature gave a higher selectivity for the carotenoids.

Modifiers are added to an extraction process mainly for two reasons [34]: (i) to increase the polarity of the SC-CO_2 in order to improve the solubility of the analytes, as mentioned above; and (ii) to facilitate desorption of analytes from the sample matrix. The small polar modifier molecules accelerate desorption processes by competing with the analytes for the active binding sites, as well as by disrupting matrix structures. For example, the effects of ethanol addition have been investigated for the extraction of β-carotene from carrots [32,33,48] and from tomato paste waste [59], and in most cases increasing modifier concentration of the SC-CO_2 resulted in increasing β-carotene recoveries. Similarly, Chandra and Nair [36] noted that SC-CO_2 containing 5% chloroform and 5% hexane gave 93 and 77% recovery of carotenoids from carrots, respectively, compared to 66% recovery when no modifier was added.

There are however two major drawbacks in using modifiers. The selectivity for a specific analyte usually decreases, resulting in coextracted solutes which can also complicate analyte collection [60]. If a solid-phase trap is used for collection, its temperature must be high enough so that the modifier will not condense in the trap and cause breakthrough of the analytes off the trap [61,62]. An alternative is to

use a longer trap of higher capacity for condensed modifier, thereby allowing the use of lower temperature [63]. For solvent collection, it is important that the modifier be compatible with the collection solvent to avoid formation of two-phase systems.

The extraction times for both static and dynamic modes should also be selected with respect to the sample type. As noted above, desorption-controlled matrices are gained by a relatively long static extraction mode, while solubility-controlled matrices are rather unaffected by the static extraction mode. A straightforward way of optimizing the dynamic extraction time is to expose the sample to a long dynamic extraction step with fractionated collection in separate vials at fixed time intervals. In this case, it is important to apply a sufficiently long dynamic extraction time, since the final phase of the extraction can be slow resulting in a small fraction of the analyte still residing in the matrix [45].

2.2.4. Collection

There are basically two different ways of collecting the analytes and the coextractives [64]: into a solvent [65–67] (Fig. 1A) or onto a solid-phase trap (Fig. 1B). The collection mechanism for the latter is based on adsorption aided by cryogenic cooling [68,69] or cryogenic cooling solely [70,71]. Tandem traps, incorporating a solid-phase trap followed by a vessel containing a solvent, have also been used [72,73]. It is also possible to collect the extract directly in an empty glass tube [74], but this is not advisable for analytical determination of vitamins due to the high risk of oxidative degradation. However, this type of collection is common for preparative enrichment of fat-soluble vitamins, e.g., when the extract will be used as colorant (carotenoids) or as antioxidant (tocopherols) [58,75].

Collection in a solvent is achieved by simply keeping the end of the restrictor in a glass tube filled with solvent. The collection parameters to be considered are (i) the solvent type; (ii) the collection temperature; (iii) the restrictor flow-rate; (iv) the solvent volume; and (v) the restrictor temperature. In Table 2, the importance of proper choice of collection solvent for the determination of vitamins A and E in milk powder is demonstrated (unpublished data).

The results in Table 2 show that a mixture of ethanol and diisopropyl ether (EtOH–DIPE, 1:1, v/v) gives higher recoveries compared using either pure isopropanol (IPA), methanol (MeOH) or acetone. The superior collection efficiency of this solvent mixture is most likely due to its ability to dissolve larger amounts of coextracted fat.

Table 2
Recoveries of retinol and α-tocopherol from milk powder using different collection solvents ($n=2$)

Collection solvent	Retinol recovery (%)	α-Tocopherol recovery (%)
EtOH–DIPE	102	85
IPA	108	68
MeOH	97	55
Acetone	89	42

The temperature of the solvents was 10°C, their volumes were 15 ml and the restrictor flow-rate was 1.0 ml/min.

McDaniel et al. [67] performed factorial designed experiments to investigate the effects of collection solvent, restrictor flow-rate, collection temperature, restrictor temperature and pressurization of the collection vessel on the collection efficiency of fat-soluble vitamins. It was found that the collection solvent had largest effect on successful collection. For example, recoveries for vitamins A, D, E and K increased when changing from hexane to ethanol and from ethanol to isopropanol. It was also shown in this study that lowering the collection temperature resulted in increasing recoveries of vitamins A, D and E. This trend was rationalized by invoking the decreasing volatility of the solvent as well as the increasing viscosity of the solvent.

Collection on solid-phase traps is achieved by depressurizing the supercritical fluid at the entrance of a column packed with an inert or adsorbing material. The trapped analytes are normally eluted afterwards in one fraction into a vial. The main parameters to optimize are (i) type of packing material; (ii) the trap temperature; (iii) the rinse solvent; and (iv) the trap geometry.

A variety of different adsorbing packing materials have been used for collection in SFE. These include charcoal [76], octadecyl silica (ODS) [34], diol and silica [68], silica gel [72], Florisil [77], Tenax [78], and alumina [78]. Analytes can also be trapped cryogenically on beads of stainless steel or glass [68,73], or cryogenically focused on stainless steel

surfaces [79,80] or in a fused-silica capillary [70]. If SFE is coupled on-line to SFC, a trap column is commonly employed in which the pressure is kept lower than the extraction pressure, thus resulting in deposition of the extracted solutes at the beginning of the column [81–83].

There are only a few applications in which solid-phase traps have been used for collection of fat-soluble vitamins [34,38,52,63,82,84–86]. The packing materials employed were silica gel [82,84], stainless steel beads [34,86], and ODS [34,38,63,85]. Marsili and Callahan [34] found that ODS was superior compared to stainless steel beads in trapping β-carotene using SC-CO_2 modified with ethanol.

It is important that the trapping temperature be kept low enough so that analytes are properly trapped and not degraded. Recently, Eskilsson et al. [63] have demonstrated that instead of applying a high trap temperature in order to avoid breakthrough losses of fat-soluble vitamins, that a longer trap with higher collection capacity is feasible. Such a trap enabled quantitative collection of vitamins A, D and E at moderate trapping temperature (60°C) when 4% of methanol was used as modifier with SC-CO_2. Such a long trap also permits fractionation of the vitamins from coextracted fat during the subsequent elution procedure to recover the fat-soluble vitamins.

2.3. Applications

2.3.1. Vegetables

Carotenoids have been extracted and enriched mainly from carrots [32–34,36,48], but also from sweet potatoes [31], paprika [58], tomato paste waste [59] and mixed vegetables such as broccoli, collard greens, corn and zucchini [34].

Spanos et al. [31] investigated the effects of pressure and temperature on the extraction of β-carotene from sweet potatoes, and found that the recovery increased when the pressure was increased at constant temperature, such increment being more pronounced at higher temperatures. When temperature was increased at constant pressure, the recovery increased at pressures above the crossover point, but decreased at pressures below the crossover point. This crossover point was found to be around 20–25 MPa. Baysal et al. [59] also found an increasing recovery of β-carotene from tomato paste waste with increasing temperature at constant pressure (300 bar). Temperatures of 35–65°C were investigated in this study, and the highest yields were achieved at 65°C. The effect of extraction pressure demonstrated that at the highest pressure investigated (300 bar) the highest β-carotene recovery was achieved. As mentioned in Section 2.2, increasing pressure gives a higher solvent density, and therefore increases the extraction rate. However, results by Barth et al. [33] indicate lower extraction yields of α- and β-carotene from carrots using pressures of 400 atm and above. Similarly, Chandra and Nair [36] recorded enhanced recoveries of α- and β-carotene when increasing the extraction pressure from 40 to 50 MPa, but the carotenoid recoveries decreased when the pressure was further increased to 60 MPa. Chandra and Nair invoked the increasing polarity of the supercritical fluid when the density was increased from 1.04 to 1.34 g/ml at 40°C to explain such trends. As noted previously, maximum solubility of a solute in a supercritical fluid is achieved when the solubility parameter of the solute and the fluid are equal. The solubility parameter of carotene is 8.71 $cal^{1/2}/cm^{3/2}$ and for SC-CO_2 at 40°C and 50 MPa 8.66 $cal^{1/2}/cm^{3/2}$ [57], i.e., almost the same (1 cal=4.184 J). However, at higher pressures, e.g., at 70 MPa and 40°C, the solubility parameter of SC-CO_2 is approximately 9.12 $cal^{1/2}/cm^{3/2}$ [57].

In the often-cited studies of Marsili and Callahan [34], the advantages of using ethanol as modifier in the extraction of α- and β-carotene from various vegetables is demonstrated. Here, it was found that the solubility of the carotenes in SC-CO_2 was 200 times higher when 100 μl of ethanol was added to a 200-mg sample of crystalline β-carotene, relative to the results found with neat SC-CO_2. Consequently, real vegetable samples were blended and homogenized with 2 g of ethanol, and mixed with Hydromatrix drying agent, and extracted with SC-CO_2 at 40°C and 338 atm using 20-min static followed by 10-min dynamic extraction time. The obtained carotene recoveries were comparable to those obtained using conventional solvent extraction.

Chandra and Nair [36] instead employed continuous addition of hexane or chloroform to the SC-CO_2 for the extraction of β-carotene from carrots, and found that SC-CO_2–chloroform (95:5) gave significantly higher recovery than found with SC-CO_2–

hexane (95:5). These recorded recoveries yielded significantly higher recoveries than using neat SC-CO_2.

However, both hexane and chloroform should be avoided due to the reported health hazards and negative environmental impact [87]. Therefore, ethanol has more frequently been used, to improve the extraction yield of carotenoids from carrots [32,33,48] and other vegetable matrices. For example, Vega et al. [32] showed that the addition of ethanol has a considerable effect on the recovery of carotenoids from carrots; 10% continuous modifier addition resulting in 100% analyte recovery regardless of the extraction temperature utilized (40–70°C investigated).

Extraction time and flow-rate as parameters have not received as much attention as temperature, pressure and modifier. However, Baysal et al. [59], demonstrated that to long an extraction time resulted in an actual lower recovery of the carotenoids, supposedly due to degradation. Also the fluid flow-rate was shown to have an optimal value, which the authors vaguely explained citing a change of mixing conditions in the extractor. A more probable explanation is that the extraction process of carotenoids from tomato paste waste is desorption-limited, which adversely affects the extraction recovery when higher flow-rates are applied using the same amount of extraction fluid.

2.3.2. Plants and oilseeds

SFE has been applied to extract and enrich tocopherols and carotenoids from both plants and oilseeds [35,56,57,75]. Tocopherols and carotenoids have also been determined as a measure of quality in oils extracted from hiprose fruit [30], mille thistle fruit [88], rapeseed, sunflower and soybeans [89] and coriander seeds [90]. In addition, SFE coupled to preparative SFC has been applied for the enrichment of tocopherols from cereals [81,83].

Favati et al. [57] showed that by optimizing the pressure, a high degree of selectivity could be obtained for the extraction of β-carotene and lutein from leaf protein concentrates. At 30 MPa and 40°C, the yield of carotene was over 90%, while that of lutein was below 30%. Colombo et al. [75] found that the highest pressure they investigated (24 MPa), yielded the highest recovery of tocopherols and tocotrienols from barley. The same trend was found by Birtigh et al. [56] for the enrichment of carotenoids from palm fruits and tocopherols from palm leaves. However, at higher pressures the selectivity with respect to triacylglycerols in the samples decreased, leading to a lower vitamin concentration in the extracts.

In two similar studies by Matthäus and Brühl [89,91], the tocopherol concentration determined in oils extracted from sunflower seeds, rapeseeds and soybeans, were used as an indicator of possible alteration of the oil. The effect of extraction temperature on the recovery of tocopherols, free fatty acids (FFAs), diacylglycerols (DAGs) and total fat were investigated in this study, revealing a general increase in recoveries for all the investigated components up to 75°C, followed by a decrease in recovery for tocopherols, FFAs and DAGs at 100°C. However, no precision indices (relative standard deviations, RSDs) values were given, making interpretation of the results difficult. The results using SC-CO_2 at 100°C and 52 MPa were compared with those obtained using other extraction methodologies, including the German Society for Fat Science (DGF, Deutsche Gesellschaft für Fettwissenschaft) standard method B-I 5 (87) (shaking with solvent), accelerated solvent extraction (partition into hot, pressurized solvent), Soxtherm (soxhlet-type of extraction) and FexIKA 200 (modified soxhlet-type of extraction). The highest tocopherol recoveries were achieved, however, using the SFE-based methodology, while the obtained results for total fat content were similar for all the extraction methods. Thus, the mild extraction conditions in SFE most probably keeps tocopherol degradation at a low level.

Illés and co-workers [30,90] determined tocopherols and carotenoids in the oil extracted from hiprose fruit [30] as well as tocopherols in the oil extracted from coriander seeds [90]. Supercritical and subcritical CO_2, propane and mixtures of both were used as extraction fluids. In these studies, the experimental results showed that 10–20 times less of SC-CO_2–propane (1:2, w/w) mixture was needed for the quantitative extraction of oil from hiprose fruit and coriander seeds, when compared to neat SC-CO_2. In addition, milder conditions (i.e., lower extraction temperature and pressure) could be applied using the SC-CO_2–propane mixture relative to

using neat SC-CO_2. Furthermore, the concentration of tocopherols and carotenoids were higher in those oil extracts obtained using SC-CO_2–propane as fluid. Interestingly, subcritical CO_2 gave higher tocopherol recovery than SC-CO_2. However, one advantage of using neat SC-CO_2 was the possibility of obtaining fractionation between essential oils and the rest of the lipids in the coriander seed oil.

Saito et al. [81] extracted and enriched tocopherols from wheat germ employing an on-line SFE–preparative SFC method. SC-CO_2 at 250 bar and 40°C was used for the extraction step while SC-CO_2 containing ethanol at 300 bar was used for preparative SFC. Using this coupled technique, a 100-fold enrichment of tocopherols was obtained, and another 4-fold enrichment was obtained by recycle preparative SFC of consecutive fractions. In a similar study by King et al. [83], tocopherols were extracted and enriched from soybean flakes and rice bran. Here, it was discovered that the extraction of oil required less SC-CO_2 per gram of substrate employing higher pressures and temperatures, such as 70 MPa and 80°C. However, fractionation and enrichment of tocopherols from soybean flakes or rice bran was preferably achieved by applying a lower extraction pressure, 25 MPa. It was found that tocopherols were extracted in the early SFE fractions, and that when these fractions were subjected to preparative SFC at 25 MPa and 40°C, that a 12-, 15- and 30-fold enrichment was achieved for α-, γ- and δ-tocopherol in soybean flakes, respectively. Interestingly, β-tocopherol was not enriched in these studies.

2.3.3. Dairy and meat products

Three analytical applications by Schneiderman et al. describe the determination of vitamin K_1 (phylloquinone) in infant formula [84], vitamin K_3 (menadione) in animal feed [92] and vitamin A palmitate (retinyl palmitate) in cereal products [85]. Only spiked samples were used in these studies, and no optimization of extraction parameters was done. SC-CO_2 at 55 MPa and 60°C gave quantitative recoveries after only 20 min of static extraction and simple depressurization into a solid-phase trap.

In another analytical study, Burri et al. [52] applied SFE for the determination of β-carotene in vitamin tablets and retinyl palmitate in calf liver tissue. SC-CO_2 at 31 MPa was used at 40°C for the vitamin tablet assay and 31 MPa and 80°C for the liver samples. In these extractions, a 1-min static extraction time was followed by 40-min dynamic extraction interval using a flow-rate of 2 ml/min. The obtained vitamin recoveries were identical or better than those obtained employing conventional solvent extraction methodology.

Turner and Mathiasson [37] have developed an analytical methodology for vitamins A and E in milk powder based on SFE followed by saponification. Several parameters were investigated, including collection solvent and temperature, entrainer addition, extraction temperature, restrictor flow-rate and extraction time and mode. It was found that the addition 2 ml of an alcoholic entrainer, such as ethanol or methanol, prior to SFE was critical to achieving quantitative recovery of the vitamins, together with the continuous addition of methanol to the supercritical fluid (SC-CO_2–methanol, 95:5). When the initial 2-ml alcoholic entrainer was excluded, but the same supercritical fluid was used, the obtained vitamin recoveries were below 60%. The optimized SFE method employed SC-CO_2–methanol (95:5, v/v) at 37 MPa and 80°C, a 15 min static time followed by 15 min dynamic extraction at a flow-rate of 1.0 ml/min. Collection of the extract was achieved in 16 ml of ethanol–diisopropyl ether (1:1, v/v) at 5°C. Vitamin recoveries based on comparison with conventional solvent extraction were 99 and 96% for vitamins A and E, respectively, and RSD values were 5 and 8% for vitamins A and E, respectively.

In a similar study by Berg et al. [38], based on the above methodology [37], vitamins A and E were determined in milk powder, milk, minced meat and liver paste. Two similar methodologies were applied on two different automatic SFE-instruments, employing solvent collection (Isco SFX 3560) and solid-phase trapping (HP 7680T). Extraction results from this study were compared with those obtained using conventional solvent extraction. A fractionated extraction/elution scheme was applied in the solid-phase trap experiment, in order to avoid analyte breakthrough losses due to the large amounts of coextracted fat. Extraction parameters for solvent collection experiment, was identical to those in Ref. [37], with the exception that a dynamic extraction time of 60 min was employed. The extraction

parameters for the solid-phase trap extraction experiment were SC-CO_2–methanol (96:4, v/v) at 37 MPa and 80°C, a 5-min static extraction followed by a 42-min dynamic extraction at a flow-rate of 1.0 ml/min. The ODS-trap temperature was set to 90°C during the extraction, and desorption of the trap was achieved with *n*-hexane–dichloromethane (1:1, v/v) after 1.25, 20 and 20 min of dynamic extraction. Agreement between the results obtained using conventional solvent extraction and SFE-based methodology employing two different types of equipment was quite good.

In a continuation of the methodologies described above [37,38], the harsh saponification step was replaced by enzymatic hydrolysis to enable simultaneous extraction and hydrolysis of fat and vitamin esters [53,54]. In this case, immobilized lipase was loaded into the outlet side of the extraction cell as shown in Fig. 3, and the hydrolysis reaction took place under supercritical conditions.

Several immobilized lipases were tested, of which Novozyme 435 (immobilized *Candida antarctica* lipase type B) showed the best performance for catalyzing the hydrolysis of retinyl esters to retinol. Parameters such as pressure (2500–7000 p.s.i.; 1 p.s.i. = 6894.76 Pa), temperature (40–80°C), modifier (1, 2 and 5%, v/v, of ethanol) and extraction time (up to 60 min dynamic extraction) were optimized to achieve highest yield for the extraction–enzymatic reaction of vitamin A from milk powder. The optimal parameters were found to be SC-CO_2–ethanol (95:5, v/v) at 260 bar and 60°C, a 15-min static extraction followed by a 45-min dynamic extraction at 0.5 ml/min SC-CO_2 flow-rate. These supercritical conditions gave similar results or better for the determination of vitamins A and E in milk powder, infant formula, low-fat and high-fat liver paste and minced pork and beef meat, when compared to conventional methodologies based on saponification and solvent extraction. The SFE method using the enzymes was concluded to be faster, more amenable to automation and consumed less amounts of organic solvents than either conventional solvent extraction or SFE with off-line saponification. The general use of enzymes in supercritical systems has been described in several excellent review papers [93–95].

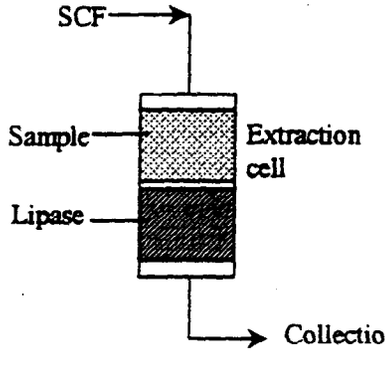

Fig. 3. Illustration of an extraction cell filled with one layer of sample and one of immobilized lipase used for catalyzing on-line SFE–enzymatic hydrolysis.

2.3.4. Pharmaceuticals and cosmetic creams

Scalia et al. [47] determined retinyl palmitate and tocopheryl acetate in four different pharmaceutical tablet preparations using SC-CO_2 extraction. Pressure, temperature and extraction time were optimized to 250 atm, 40°C and 15 min dynamic extraction, respectively. The developed SFE methodology was compared to conventional solvent extraction, which revealed no significant difference between the obtained results. Moreover, the selectivity with respect to high-molecular mass carbohydrates and fat was higher with the SFE-based methodology than with conventional methods. The recoveries for the four tablet preparations were between 97.8 and 110.1% when compared to the label content and the RSD values ranged between 1.5 and 3.9%.

Similarly, Scalia et al. [96], determined retinyl palmitate and tocopheryl acetate in cosmetic creams and lotions employing SFE followed by HPLC analysis. Here it was shown that extraction pressure and the degree of sample dispersion mainly affected the recoveries. An extraction pressure of 250 atm was found to give higher solubility of the vitamin esters than using a pressure of 200 atm at 40°C. Thorough dispersion of the sample (0.10–0.15 g of cream) with Celite, was critical to achieve high extraction recoveries, i.e., application of the cream on filter paper resulted in recoveries below 20%. Using a restrictor temperature of 60°C resulted in plugging, but this problem was solved by increasing the restrictor temperature to 100°C. SC-CO_2 at 250 atm and 40°C, and an extraction time of 30 min, gave recoveries between 91.6 and 94.9% for the vitamin esters in four commercially available cos-

metic creams. The RSD values for these determinations ranged between 3.9 and 6.1%.

Gámiz-Gracia et al. [86] determined vitamins D_2 and D_3 in various pharmaceutical preparations, including two types of water-based drops, one powder and one granulated. The collection during SFE was achieved on a trap filled with stainless-steel beads, which after extraction were rinsed with methanol. Spiked diatomaceous earth was used for optimization of the extraction parameters, including temperature (40–80°C), density (0.60–0.95 g/ml), flow-rate (0.5–2.5 ml/min), extraction time and mode, entrainer (0.15–1.5 ml of toluene, methanol and diethyl ether) and sample size (0.5–2.0 g). The optimized conditions were SC-CO_2 at 40°C and 281 bar (0.90 g/ml), and 1 min static extraction followed by 20 min dynamic extraction at a flow-rate of 2.0 ml/min, 0.5 g of sample, and 0.25 ml of diethyl ether addition to the sample 10 min before SFE. The addition of diethyl ether improved the vitamin D recovery, toluene had no effect on the recovery, and methanol gave even lower recovery. The trap temperature during the extraction was not given, which makes it difficult to interpret the negative results with methanolic entrainer. The use of stainless-steel beads for trapping only relies on cryogenic cooling, which makes condensation of coextracted modifier quite plausible, and this would lead to fast breakthrough of the target substances. The recoveries for the different pharmaceutical preparations were between 85 and 105%, and the standard deviations ranged between 2 and 12%.

Becerra et al. [97] employed SFE followed by SFC for the determination of vitamin E in four pharmaceutical preparations. Two of the preparations were spiked samples (tablet and dietetic milk powder) and two commercially produced tablets. Several extraction parameters were optimized, and for vitamin E the effects on recovery were largest when changing pressure or temperature. The highest pressure employed, 400 atm, at 60°C gave the highest recovery. Extraction temperatures between 40 and 70°C were investigated at the same constant pressure (400 atm), which resulted in a bell-shaped curve of recovery versus temperature, with an optimal temperature of 60°C. The flow-rate (1–4 ml/min tested) did not affect the vitamin recovery significantly, hence 2 ml/min was chosen for a supercritical fluid flow-rate. The dynamic extraction time was optimized to 30 min, because extraction times below 25 min gave lower recovery for most of the pharmaceutical preparations. The SFC methodology utilized a column temperature of 60°C, and progressive increases in pressure from 100 to 450 atm. A packed column (Nucleosil C_{18}) was used, and detection was made by coupling the fused-silica restrictor to a FID system. Recoveries for the coupled method were between 94 and 102%, with standard deviations ranging between 0.7 and 7%.

3. Supercritical fluid chromatography

SFC embraces many of the features of liquid and gas chromatography, and occupies an intermediate position between the two techniques. In most applications, SC-CO_2 is used as the mobile phase, having many of the same advantages discussed for SFE (see Section 1.1). Moreover, SC-CO_2 is UV transparent and gives minimal response when using FID. A simple flow schematic of typical SFC instrumentation is shown in Fig. 4.

There are two major forms of SFC depending on which type of column is employed: open-tubular SFC (OT-SFC, see Fig. 4A) and packed column SFC (PC-SFC, see Fig. 4B). OT-SFC has some similarities to capillary GC, and PC-SFC employs modified HPLC instrumentation. The internal diameters of the capillary columns are smaller in SFC than in GC to compensate for the lower solute diffusion rates in the mobile phase, and thereby maintaining the desired high column efficiencies. Because of these smaller inner diameters as well as the ultra-thin stationary film thickness employed in OT-SFC, the injection volumes are smaller in OT-SFC than in capillary GC.

Preferably two high-pressure pumps (for delivery of supercritical fluid and modifier) are employed to deliver mobile phase to the system. A restrictor is placed either before the detector (detector operating at ambient conditions, e.g., FID system, see Fig. 4A) or after (a pressurized detector module, e.g., UV, see Fig. 4B). A restrictor placed after the detector requires that a high-pressure cell be employed in the SFC instrumentation. SFC is compatible with detection methods used in both GC and LC, such as

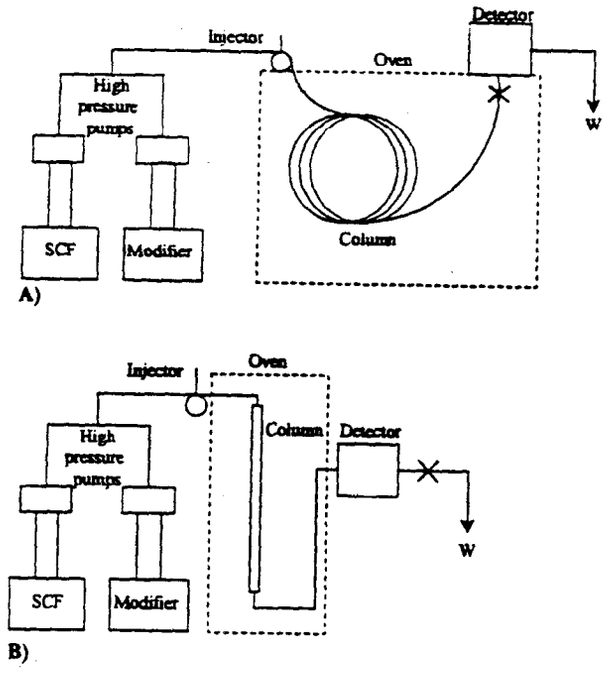

Fig. 4. A flow schematic of two different types of SFC equipment: (A) OT-SFC with a detector cell at ambient condition and (B) PC-SFC with a pressurized detector cell. Dashed lines indicate temperature-controlled zone (oven).

FID, UV–Vis, DAD, fluorescence, IMS, MS, ECD, FT-IR, ELSD and even NMR. However, in fat-soluble vitamin determination, UV, FID and MS detectors have generally been employed. More information regarding instrumentation in SFC can be found in the excellent book by Lee and Markides [21] and in various review papers [14–17].

3.1. Important parameters affecting retention in supercritical fluid chromatography

Important parameters affecting retention in SFC are those which control (i) the solvent strength of the supercritical fluid, and thereby the partition coefficient of the solute, (ii) peak-broadening during the elution process, and (iii) interaction of the solute with the stationary phase. Some basic theory on chromatography is described in short below. For more thorough information the reader should consult Refs. [20,21].

3.1.1. Theory on chromatographic retention

Solutes from the injected plug are distributed between the mobile phase (the supercritical fluid) and the stationary phase according to the partition coefficient of the solute. When the peak reaches the detector, it has a certain width depending on various peak-broadening effects, for example caused by different types of diffusion of the solutes in the mobile phase and interactions of the solutes with the stationary phase. Generally the van Deemter equation is applicable to SFC, showing the dependence of the height equivalent of a theoretical plates (H) on the average flow-rate (ν_0) of the mobile phase:

$$H = A + B/\nu_0 + C_m\nu_0 + C_{st}\nu_0 \quad (2)$$

where A is the Eddy diffusion (arising from the different path lengths encountered by solutes in packed columns, which therefore equals zero in capillary columns), B arises from the longitudinal diffusion of the solute in the mobile phase, C_m arises from the velocity gradient in the mobile phase affected by the radial diffusion, and C_{st} arises from the interactions of the injected solute with the stationary phase. Depending on effects of diffusion and flow-rate, there is an optimal (minimal) value of H at a certain average flow-rate (ν_0) of the supercritical fluid. At this flow-rate, the column has its highest possible number of theoretical plates (N) under the selected conditions, i.e., highest separation efficiency.

The resolution (R_s) of two peaks is a function of the separation efficiency (described by N), the separation selectivity (α), and the retention factor (k):

$$R_s = 1/4N^{1/2}((\alpha - 1)/\alpha)[k/(k + 1)] \quad (3)$$

The retention factor (previously called capacity factor) describes the retention of a peak relative to that of the mobile-phase plug. The separation selectivity is the quotient of the retention factors of two adjacent peaks. In general, a resolution of 2 is considered to be acceptable to enable quantification of the peaks [20].

3.1.2. Pressure and temperature

As already discussed above for SFE (see Sections 2.1.1 and 2.1.2), pressure and temperature together

determine the solvent strength of the supercritical fluid. Moreover, at constant pressure, there is a solubility-minimum at a certain temperature (referred to as the "crossover point" in SFE, see Section 2.1.2). At this point, the retention factor reaches a maximum. The solubility is inversely linearly proportional to the retention factor, and thereby the retention volume. Hence, retention in SFC has been used for providing a measure of the solubility of a solute in supercritical fluid at different conditions.

3.1.3. Modifiers

It has already been discussed previously how an organic modifier, such as methanol, can be added to the supercritical fluid to increase its polarity (see Sections 2.1.3 and 2.2.3). The use of modifiers however in SFC can strongly affect the retention of analytes. In addition, the smaller modifier molecules can interact with residual silanol groups of the stationary phase, which thereby shortens the retention time of solutes and improves their peak shape. A modifier may also alter the elution order of solutes, as can a change in mobile phase density. A disadvantage with using modifiers is that FID cannot be used, due to its response to carbon-containing compounds.

3.1.4. Flow rate

The flow-rate of the supercritical fluid, i.e., the velocity of the mobile phase, directly affects retention in SFC, resulting in a Van Deemter curve which is relatively flat after the minimum and allows high flow-rates without significant losses in separation efficiency. This is of interest in preparative work. For a given desired purity of a component, i.e., a given separation from other solutes, there is an optimal flow-rate (or loading of sample) that gives a maximum production rate (or minimal analysis time) [20].

3.2. Method development

3.2.1. Sample introduction

Injections in SFC are generally obtained by the use of a high-pressure valve with an internal sample loop. The injection techniques can be divided into (i) direct injection; (ii) dynamic split injection; (iii) delayed split injection; and (iv) timed-split injection [21]. The preferred injection technique depends on column type and sample amount/volume that needs to be injected, which is discussed below.

Direct injection is the ideal technique in terms of reproducibility, since the entire volume of the sample loop is injected to the column. This technique is commonly used in PC-SFC using columns having inner diameters of 0.5 mm or larger, and has widely been used in vitamin analysis [98–100]. Injection volumes in this case are typically 0.2–1 μl. However, narrow-bore and OT columns have smaller capacities for large injected solvent volumes, and other than direct injection techniques are preferred.

In dynamic split injection, a restrictor device is placed downstream of the injector. Depending on the flow-rates through the column and the split restrictor, different split ratios are obtained. Unfortunately, the relationship between the split ratio and the peak area is nonlinear. In general, dynamic split injection works better at high split ratios, but produces an inherent decrease in sensitivity of the SFC method. This injection technique is commonly utilized in applications where narrow-bore column (<0.5 mm I.D.) are employed, and gives acceptable reproducibility if the tubing connections are good and internal standards are used [101–103].

Delayed split injection functions in the same way as dynamic split injection, but with the difference that the split restrictor has an on/off valve. The valve is closed during the initial injection (typically 1–15 s), then it is opened, and a certain part of the solvent is vented out through the split restrictor. This technique is appropriate to use if larger volumes of sample are to be introduced into an OT column.

In timed-split injection, the column is connected directly to the injector valve. High-speed pneumatics and electronics controls the injector, thus permitting a certain portion of the sample loop to be introduced onto the column. The advantages of timed-split injection compared to the other split techniques, is that there is less sample discrimination, the relationship between actuation time and peak area is linear over a quite a wide range, and the amount of injected sample can easily be changed. Timed-split injection is commonly used in both OT and PC-SFC [102,104,105].

In trace analysis employing OT-SFC, some sort of solvent elimination has to be applied in order to

enable injection of large amount of sample without risking excessive band broadening and peak splitting. Solvent elimination can be achieved by employing a precolumn, from which the venting time is much shorter than from the main column. Thereafter, the sample without the solvent is transferred and focused at the beginning of the main column.

Another way of eliminating solvent is by gas purging, in which the sample is purged into a precolumn with an inert gas. The solvent is evaporated and flushed out through the vent valve, while the solutes are precipitated on the walls of the precolumn. Solvent backflush is a third approach, which is a variant of delayed split injection. The split valve is closed for about 1 min during injection, and when it is opened, a rapid negative pressure ramp is applied. The density gradient causes a backflush, resulting in solvent venting through the vent line and the solutes precipitate on the column wall.

If a supercritical fluid extractor is coupled on-line to a SFC instrument, larger amounts of sample can be introduced into the chromatographic system, as long as they are soluble in the supercritical fluid. On-line SFE–SFC approaches have been employed for enrichment of tocopherols from wheat germ [81], determination of retinyl palmitate and tocopheryl acetate in hydrophobic ointment [82] and for the analysis of a fat-soluble vitamin test mixture [106].

Besides the type of injection technique, other parameters need to be considered. For example an injector solvent should be chosen that has a high solubility in the supercritical fluid in order to obtain rapid mixing and thereby avoid the peak-splitting artifact. The injector device should be capable of being heated, in order to efficiently transfer the solutes to the column. However, injector temperature should not be too hot as this may cause boiling of the solvent with concomitant analyte loss.

3.2.2. Chromatographic separation

One of the first things to consider during SFC method development is which type of chromatographic column is optimal for the desired separation. OT columns are generally superior in terms of efficiency and inertness, while packed columns provide higher sample capacity and speed. Hence, if the sample is very complex and a large number of theoretical plates are important, OT-SFC should be selected. Additionally, if neat SC-CO_2 is to be used as mobile phase and the analytes are relatively polar, OT-SFC is preferred due to its inertness of the stationary phase. However, if large amount of sample needs to be injected, it is not appropriate to use OT-SFC because of its limitation in sample capacity (governed by the stationary phase thickness). PC-SFC on the other hand provides significantly higher sample capacity due to much larger total surface area of the packing material. In addition, many separations done via PC-SFC are generally faster than OT-SFC-based separations.

Once a column type has been selected, the next step is to choose column material and type of stationary phase. OT columns are most commonly made from fused silica, because of their ease in handling and inertness. Chemically bonded stationary phases are necessary to prevent the supercritical fluid from stripping the liquid phase from the column. Polysiloxanes containing different chemical groups, such as methyl [101,107], octyl [107], cyanopropyl [104], phenyl [104,107], mixtures of cyanopropyl–phenyl [102] and Carbowax [107,108], are common stationary phases in fat-soluble vitamin separation. For example, White et al. [109] utilized two bonded stationary phases, DB-5 (95% dimethyl–5% diphenylpolysiloxane) and DB-WAX, for the separation of vitamins A, E, D and K. Snyder et al. [107] employed SB-phenyl-5 and SB-octyl-50 for the separation of tocopherol isomers, and found that the resolution of the positional isomers β- and γ-tocopherol was somewhat improved on the SB-octyl-50 column. A more polar Carbowax column was also investigated in this study, and showed reverse elution order of the tocopherol isomers compared to the SB-phenyl-5 and SB-octyl-50 columns.

The same packed bonded-phase columns employed in HPLC can be used in PC-SFC. These are based on spherical porous silica (particle sizes 3–10 μm in diameter, pore sizes 100–300 Å). The use of silica as adsorbent in SFC leads to both reversible and irreversible adsorption of polar and medium polar analytes, especially if neat SC-CO_2 is used as the mobile phase. Therefore, it is common to use bonded stationary phases, such as octyl, octadecyl, cyanoalkyl and aminoalkyl modified silica. Unbonded silanol groups are either endcapped or used as selective adsorption sites as part of the chromato-

graphic separation. Upnmoor and Brunner [110] tested six different commercially available stationary phases for fat-soluble vitamin separation, and investigated effect of different mobile phase composition on the retention factors of the vitamins. Supercritical nitrous oxide and carbon dioxide were used in this study, modified with methanol or 2-propanol, and it was found that the retention factor decreased with increasing modifier content for all the compounds and stationary phases tested.

Packed microbore columns have found applications in fat-soluble vitamin analysis. Ibáñez et al. [108] employed a 200×0.5-mm column packed with Carbowax 20M (based on polyethylene glycol) for the separation of vitamins A, D_2, E and K_1, and evaluated the effects of varying the column pressure and temperature gradients using response contour plots. However, the vitamins eluted as only two peaks ($E+K_1$ and $A+D_2$). Hence, the Carbowax column was connected on-line to another micropacked column filled with a SE-54-based packing material in order to improve the separation selectivity [111]. The resolution of the peaks was then slightly improved.

The temperature of the supercritical fluid affects its solvent strength as well as the efficiency and selectivity in SFC. The density of the supercritical fluid is increased upon decreasing temperature, which proves useful in density programming. However, as already discussed above, the solvent strength can be either increased or decreased upon changing the temperature at constant pressure. Higher temperature results in faster solute diffusion rates in the supercritical fluid, which naturally leads to higher efficiency of the chromatographic process. Therefore, positive temperature programming is normally applied together with positive pressure programming, resulting in a compensation of the lower separation efficiency (at higher densities, i.e., pressures) with the higher diffusion rates (at higher temperatures). Note that using a higher temperature also tends to lower the solute interactions with the stationary phase. Thus, the type of stationary phase is important to consider in this context, since a good stationary phase should provide a high selectivity and exhibit a high efficiency over a wide range of temperatures.

Usually, a low supercritical fluid density is applied initially in the chromatographic run, in order to amplify large differences in solute solubility between the most retained and the least retained solute. Then the mobile phase density is increased linearly, somewhat analogous to temperature programming in GC. Density programming is easier to optimize than pressure programming, because prediction of the solute retention time is easier. Density programming is obtained either by positive pressure programming or negative temperature programming. Pressure and temperature programming can also be combined, even though prediction of retention then is more difficult. Ibáñez et al. [108] optimized pressure and temperature gradients for the separation of vitamins A, D_2, E and K_1 employing micropacked columns, and found highest separation and efficiency using programming rates of 4 atm/min and 7°C/min, respectively. In studies by Snyder et al. [107], tocopherol isomers were separated on a SB-octyl-50 column employing a positive pressure program (100–350 atm) coupled with positive temperature programming (100–180°C), directly followed by a negative temperature programming (180–100°C). Asymptotic density programming is applied to obtain equally spaced peaks, which is common in polymeric oligomer separations. This principle was utilized by Schmitz et al. [104] to separate carotenoid isomers in tomato extracts on a SB-phenyl-50 column.

The use of high-applied column pressure in SFC leads to large pressure drop over the column. However, the pressure drop over packed columns in SFC is much smaller than in HPLC, due to the lower viscosity of a supercritical fluid compared to that of a liquid mobile phase. Therefore, several coupled columns, or a relatively long column can be used to obtain a larger number of theoretical plates. For example, Berger and Wilson [112] used 10 serially connected 200-mm packed columns for the determination of lemon oil components. It was shown that 250 000 plates could be obtained in this case using nonprogrammed PC-SFC.

Advantages and disadvantages of using modifiers in SFC have been discussed above. Several different types of modifiers have been used in fat-soluble vitamin separations, such as methanol [100,110], ethanol [104], mixtures of methanol and acetonitrile [113], and 2-propanol [110]. Yarita et al. [100] investigated the effects of methanol concentration in the supercritical fluid on the retention of tocopherols

in an ODS-silica gel column. It was found that a small amount of methanol was needed to improve the peak shapes (and thereby also the resolution), and that further increases in the methanol concentration, resulted in decreasing retention factors and decreasing resolution between the individual isomers. The highest resolution between β- and γ-tocopherol was found using 0.5% (v/v) methanol in SC-CO_2.

The flow-rate of the mobile phase also needs to be optimized, in order to obtain good separations commensurate with short analysis times. Matsumoto et al. [98] showed that packed capillary SFC–MS could be employed for the analysis of water and fat-soluble vitamins, and that retention factors were nearly unaffected at fluid flow-rates in the range of 20–90 μl/min when the column was hold at constant pressures. Hence, the change in pressure drop over the column was small regardless of the flow-rate, due to the large particle size used for packing the capillaries (30–50 μm).

3.2.3. Detection

FID is the most commonly used detection method in SFC. The organic compounds decompose and ionize in an H_2–air or H_2–O_2 flame. The detector is most commonly employed with OT-SFC [107,109], but has also been used with micropacked column SFC [108] and packed capillary SFC [103].

UV–Vis detectors respond to solutes absorbing light, and are commonly employed with PC-SFC [110,114,115]. In contrast to FID, UV–Vis detection is nondestructive and offers selective detection of solutes. Moreover, modifiers can be used in a wide range of wavelengths. However, all closed cell detectors (HPLC type) suffer from slightly lower sensitivity compared to in HPLC, because of the shorter path lengths and as mentioned previously the smaller injection volumes. Note that the λ_{max} for a solute is different in different organic solvents as well as in supercritical fluid of different densities. This was revealed in a study by Hui et al. [115], in which shifts in λ_{max} were observed in the determination of carotenoids using UV–Vis detection. For example, λ_{max} was slightly higher in SC-CO_2 than in hexane. In addition, λ_{max} increased linearly with increasing density of the SC-CO_2, showing a λ_{max} of 430 nm at 0.7 g/ml and 436 nm at 0.95 g/ml for all-*trans*-β-carotene.

DAD is based on fast wavelength scanning over a selected range of wavelengths, thereby providing with full spectral information for each eluting fraction of a peak. Hence, the use of DAD facilitates the identification of an analyte, similar to in HPLC–DAD.

MS detectors are increasingly being employed with SFC. Most quadropole instruments can handle flow-rates from OT-SFC. However, for PC-SFC an interface such as thermospray or moving belt has to be employed, unless micropacked columns are used. MS detection has been used in several fat-soluble vitamin applications [98,99,107]. Matsumoto et al. [99] used semi-micro packed column-SFC coupled with API-MS detection (atmospheric pressure ionization MS) for the analysis of various test compounds, including fat-soluble vitamins. Parameters such as nebulization temperature, desolvation chamber temperature, position of the nozzle tip, flow-rate of the nebulizing gas and drift voltage were optimized to yield optimal detector performance.

Fluorescence detection is a more selective and sensitive technique for fluorescing analytes compared to FID and UV. However, fluorescence detection has not been widely employed in SFC because of the difficulty in constructing high-pressure cells without loosing attendant sensitivity. IMS systems provide a more general detection method, and can be thought of as an ECD system to which an ion separation chamber has been connected [21]. IMS detection has been employed in OT-SFC of vitamin E from vitamin capsules [116]. NMR spectroscopy can also be coupled to SFC as a powerful tool for the determination of *cis/trans* isomers. This has been shown in a work by Braumann et al. [117], in which ^{1}H NMR was coupled to PC-SFC for the determination of five different retinyl acetate isomers, yielding two-dimensional chromatograms within 25 min.

3.3. Applications

3.3.1. Carotenoids

OT and PC-SFC can be employed for the separation of *cis/trans* isomers of carotenoids. For example in a work by Schmitz et al. [104], carotenoids were extracted from carrots and separated by OT-SFC using SC-CO_2 containing 1% (v/v) ethanol.

β-Carotene *cis/trans* isomers were separated on a SB-cyanopropyl-25-polymethylsiloxane column, while α-carotene *cis/trans* isomers were separated on two SB-cyanopropyl-50-polymethylsiloxane columns. The developed SFC methods gave better separation of the carotenoids when compared to an isocratic non-aqueous reversed-phase HPLC method.

In two subsequent publications by Aubert et al. [114] and Lesellier et al. [113], *cis/trans* isomers of α- and β-carotene were separated by PC-SFC. Effects of temperature, pressure and modifier composition on the chromatographic retention was investigated and characterized by solute retention factor, peak resolution, selectivity and the number of theoretical plates [114]. Ternary mixtures of SC-CO_2, methanol and acetonitrile were used, in which the volume percentage of SC-CO_2 was set to 85%, while the composition of the organic modifier was varied. The supercritical fluid composition significantly affected the selectivity, while the effects of temperature and pressure on the selectivity were minimal. However, temperature and pressure did affect the efficiency of the separation, and an optimal temperature was found to lie between 22 and 25°C. In the subsequent publication 22 different bonded stationary phases were investigated with respect to the chromatographic retention [113]. In these studies, the retention factor for α-carotene had to be above 6 to obtain sufficient separation of α- and β-carotenes. This was achieved on a column with a high carbon loading, such as Suplex pKb-100. For the separation of *cis* and *trans* isomers of α- and β-carotenes, the modifier concentration could not be higher than 5 or 10% (v/v).

3.3.2. Tocopherols

Applications of tocopherol analysis employing SFC include preparative work [81,118], characterization of lipid components from onion seeds [101], sea buckthorn and cloud berry seeds [102] and marine oils [105], and analysis of antioxidant samples and deodorizer distillates [107], vegetable oils [100] and pharmaceutical preparations [97].

Saito et al. [81] employed SFE on-line coupled to semipreparative PC-SFC for enrichment of tocopherols from wheat germ. In this case, the extraction pressure was 250 bar, and the separation pressure was 300 bar. A silica gel column (200×20 mm) was employed as separation column, and ethanol was used as modifier in the mobile phase. The original level of tocopherols in the wheat germ powder was 0.05% (w/w), after SFE it was 0.4% (w/w) and after SFC 4–6% (w/w), i.e., a 100-fold enrichment. In subsequent studies, Saito and Yamauchi [118] further enriched α- and β-tocopherol from the wheat germ oil employing recycle semipreparative PC-SFC. The final concentration after recycle-preparative SFC was 85 and 70% (w/w) for α- and β-tocopherols, respectively.

SFC is a powerful tool in the characterization of a wide range of lipids in complex samples. Manninen et al. [102] employed OT-SFC–FID for the characterization of triacylglycerol composition and the identification of tocopherols in sea buckthorn and cloud berry seeds. A stationary phase composed by 25% cyanopropyl–25% phenyl–50% methylpolysiloxane was employed. The large loading of sample required for the identification of tocopherols resulted in overloading the column with triacylglycerols. In similar studies by Borch-Jensen and Mollerup [105], OT-SFC–FID was employed for the qualification of lipid groups, including free fatty acids, cholesterol, squalene, tocopherols, wax esters, cholesterol esters, diacylglycerols, triacylglycerols and ether lipids, from fish, shark and seal oils. TLC was initially applied to identify the different lipid groups present in the sample, and thereafter, non-polar SFC (DB-5 column) was employed for analysis of the different lipid components. Hydrogenation of samples was accomplished to give narrower peaks, and preparative TLC prior to SFC was necessary for resolving the most complex samples.

Snyder et al. [107] separated tocopherol isomers employing OT-SFC with FID and MS detection. Separation was optimized by testing four different columns, SB-methyl, SB-octyl-50, SB-phenyl-5 and Carbowax, as well as different pressure- and temperature-programming schemes (see Sections 2.2 and 3.2). The developed method was used for the determination of tocopherols in antioxidant formulations and deodorizer distillates, giving results that were in most cases higher than those obtained using conventional GC analysis. This phenomenon was explained by possible analyte losses during the derivatization step, which is necessary for the GC analysis of tocopherols.

In analytical studies by Yarita et al. [100], tocopherols were determined in vegetable oils employing PC-SFC with UV detection. The optimal modifier concentration was found to be 0.5% (v/v) of methanol in SC-CO_2, and a column pressure of 15 MPa was used. The results obtained by this SFC method showed good agreement with reference results obtained by normal-phase HPLC.

Becerra et al. [97] determined the concentration of vitamin E acetate in pharmaceutical preparations employing SFE and off-line PC-SFC–FID. A Nucleosil C_{18} column (100×1 mm I.D., 5 μm) was employed for the separation, at a column temperature of 60°C. A pressure program (100–450 atm at 70 atm/min) gave good separation. In this study it was shown that quantitative recoveries of vitamin E acetate could be obtained within 15 min of analysis time, including both SFE and SFC.

3.3.3. Separation of vitamin mixtures

Shen et al. [103] separated vitamins A, D_2, D_3, E (α-tocopherol), K_1 and K_2 on a packed capillary column (40 cm×250 μm I.D.) filled with liquid crystal polysiloxane-coated silica particles. Peak shape and retention characteristics were examined for a polar test mixture employing untreated silica, coated silica, and deactivated coated silica columns. The best peak shapes and separation as well as the shortest retention times were obtained on the deactivated coated silica column. Using this column at 70°C resulted in complete separation within 40 min of all the fat-soluble vitamins A, E, K_1 and K_2, but not complete separation of vitamins D_2 and D_3.

Choo et al. [119] fractionated crude palm oil into free fatty acids, tocopherols, diacylglycerols, triacylglycerols and carotenoids employing PC-SFC. A comparison made between a C_{18} column and a silica gel column showed better separation between the carotenoids and the triacylglycerols on the latter column. This silica gel column was used for preparative SFC of the crude palm oil, and four separate fractions were collected. The tocopherol- and carotene-rich fractions were then further purified on a C_{18} column and a silica gel column, respectively, and gave rise to peaks of considerably higher purity relative to the first preparative SFC step.

Masuda et al. [82] employed on-line SFE–PC-SFC for the determination of tocopheryl acetate and retinyl palmitate in a hydrophobic-based ointment. The SFE and SFC temperatures were 40°C and 10% ethanol was used as modifier during the SFC stage. A silica gel column (150×4.6 mm) was employed for the vitamin separation. The optimal SFC pressure was 200 kg/cm^2, which yielded good resolution (R_s=4.2) and short retention times (t_R<3 min). Spiked ointment samples were analyzed, and gave recoveries of 102% for both vitamin esters.

4. Conclusions

This review demonstrates the importance of a careful optimization of extraction and separation parameters, for the analysis of fat-soluble vitamins based on SFE and SFC, respectively. This review also points at the many advantages of using supercritical techniques instead of conventional solvent-based techniques. For example, the use of preparative SFE and SFC for enrichment of tocopherols and carotenoids from natural sources such as plants, vegetables and oilseeds, results in extracts free from organic solvent and minimally altered by thermal oxidation. This is especially useful for the production of natural additives used in functional foods and pharmaceuticals.

Analytical SFE and SFC have been used for the determination of fat-soluble vitamins in pharmaceutical preparations and cosmetic creams. Such formulations can be regarded as being similar to a spiked sample, as the vitamins are added manually to the preparation. Therefore, the extraction of these is often very fast via SFE, complete in some cases within 20 min. This makes SFE to a highly competitive technique compared to conventional solvent extraction. Several on-line SFE–SFC approaches have been demonstrated in the literature for this type of sample.

Analytical SFE applications giving recovery data from real samples (i.e., not spiked samples) are few. These include carotenoids from vegetables and plants [34,57], vitamin A palmitate and β-carotene from calf liver [52] and vitamin A and E from milk powder and meat products [37,38,53]. It is clear that the analytical determination of fat-soluble vitamins employing SFE is quite challenging, and more work needs to be done to facilitate the development of

accurate and robust methodologies. An interesting trend is the use of lipases in the extraction cell for simultaneous extraction and hydrolysis of vitamin and glycerol esters [53,54]. This achievement might attract more interest into the use of analytical SFE in fat-soluble vitamin determination, as this new enzyme-approach makes sample preparation employing SFE even faster and more amenable to automation.

Analytical SFC has several advantages over HPLC, which is the by far most commonly used analysis technique for fat-soluble vitamins. These include: (i) higher separation efficiency; (ii) smaller consumption of organic solvents; (iii) ready separation of a wider range of analytes of different sizes and polarities during one chromatographic run; (iv) separation of β- and γ-tocopherol on reversed-phase columns; and (v) separation of the *cis/trans* isomers of α- and β-carotene. Future possibilities for SFC lie within food analysis, by permitting the simultaneous determination of fat-soluble vitamins, triacylglycerols, diacylglycerols, monoacylglycerols, free fatty acids, cholesterol and other steroids and lipid esters, all in one analysis run.

5. Abbreviations

DAD	Diode array detection
DAG	Diacylglycerol
ECD	Electron-capture detection
ELSD	Evaporative light scattering detection
FFA	Free fatty acid
FID	Flame ionization detection
FT-IR	Fourier transform infrared detection
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IMS	Ion mobility spectrometry
LC	Liquid chromatography
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
OT	Open tubular
PC	Packed column
RSD	Relative standard deviation
$SC-CO_2$	Supercritical carbon dioxide
SFC	Supercritical fluid chromatography
SFE	Supercritical fluid extraction
SPE	Solid phase extraction
TLC	Thin-layer chromatography
UV	Ultraviolet
Vis	Visible

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